organic combined nitrogen. Among vertically mixed individual filaments, cellular surface to volume ratios are increased while transport to deeper, combined nitrogen–enriched waters is enhanced. In this manner, N_2 fixation is optimized as a means of obtaining combined nitrogen on calm days, whereas ambient combined nitrogen usage becomes more feasible under turbulent conditions.

Although we have shown the existence and roles of O_2-depleted microzones, the means by which intercellular "division of labor" among oxygenic photosynthesis and O_2-sensitive N_2 fixation is induced and maintained are currently unresolved. Transport of photosynthetically produced carbon compounds to O_2-depleted N_2-fixing cells must take place to provide both reductant and carbon skeletons essential as an energy source for N_2 fixation and for accepting (incorporating) recently fixed NH_3. Although transport of photosynthetically fixed $^{14}CO_2$ to internal O_2-depleted microzones can be shown by autoradiography, the genetic, physiological, and structural mechanisms that mediate such transport remain unknown.

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Translation in Mammalian Cells of a Gene Linked to the Poliovirus 5′ Noncoding Region

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The central portion (region P) of the 742-nucleotide noncoding 5′ end of poliovirus allows the RNA to initiate protein synthesis in the absence of the usual 5′ 7-methylguanosine capping group. Poliovirus 5′ noncoding region was fused to a reporter gene and transfected into cells. There was extensive augmentation of the expression of this gene by poliovirus-mediated inhibition of cap-dependent protein synthesis. That the construct initiated in a cap-independent manner was verified through in vitro experiments. Small lesions throughout region P blocked its initiation function, implying that a coherent functional unit, hundreds of nucleotides long, is responsible for cap-independent initiation by poliovirus RNA.

THE GENOME OF POLIOVIRUS IS A single-stranded molecule of plus-strand RNA, approximately 7500 bases long (*1*). Poliovirus has an unusually long 5′ noncoding region, which consists of 742 untranslated nucleotides preceding the AUG used to initiate translation (*2*). A highly significant sequence similarity extends through the first 650 nucleotides of the three poliovirus serotypes (*3*), an indication that the region has a crucial role in the viral life cycle. Poliovirus messenger RNA, unlike most other eukaryotic mRNAs, is not capped at its 5′ end; it terminates in pUp instead of the usual "capping group" $m^7G(5')ppp(5')N\ldots$ (*4*). It must therefore be translated in a cap-independent manner. The virus takes advantage of its unique style of translation initiation to inhibit cellular protein synthesis by interfering with the cap-dependent translation of cellular mRNAs (*5*).

Using a cDNA copy of the viral genome (type 1, Mahoney) (*6*), we engineered a number of mutations into the poliovirus 5′ noncoding region (*7*). The biochemical and genetic analysis of several phenotypically recognizable viral strains thereby generated showed that an RNA sequence of hundreds of nucleotides (which we called region P) is involved in allowing viral protein synthesis (*7*). In vitro experiments also suggested that a large portion of the poliovirus 5′ noncoding region is responsible for the cap-independent translation of downstream sequences (*8*). So far, no cellular gene has been

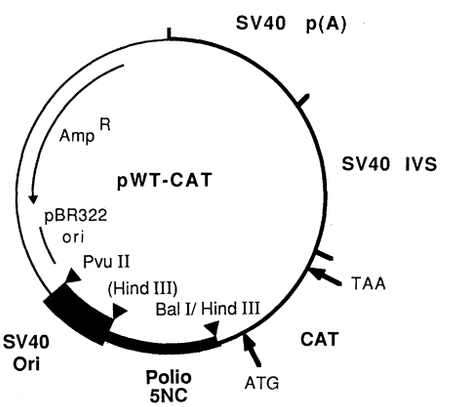

Fig. 1. Schematic representation of the wild-type construct (pWT-CAT). Nucleotides 1 to 630 of poliovirus type 1 (Mahoney) (*1*) were cloned in the Hind III site of the pSV2CAT plasmid (*9*); in the other constructs, the corresponding region of various mutated clones (*7*) was substituted for the wild-type sequence.

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shown to have such a translational module; no class of cellular mRNA that continues to be translated in poliovirus-infected cells has been detected.

We hypothesized that a gene placed downstream from the poliovirus 5′ noncoding region and introduced into mammalian cells might be translated independently of a cap structure. Not only would its translation resist the virus-induced inhibition of cap-dependent translation, but it could be enhanced in this setting. The cellular ribosomes and the other elements of the translational machinery, no longer attracted to cap-dependent mRNAs, might devote themselves entirely to the only class of mRNA still able to use them.

To test this hypothesis, we cloned the chloramphenicol acetyltransferase (CAT) gene downstream from wild-type or various mutated poliovirus 5′ noncoding regions. The engineered mRNA was inserted into a pSV2 expression vector (Fig. 1); the pSV2CAT plasmid (*9*) was used as control. All of the constructs were introduced in parallel into COS cells by electroporation (*10*). Forty hours later, a fraction of the cells was harvested for measurement of CAT activity. At the same time, another fraction was infected with poliovirus at a multiplicity of infection (MOI) of 100 in the presence of guanidine (to inhibit poliovirus replication and therefore prevent early death of the cell) and actinomycin D (to stop any further accumulation of cellular mRNA). A third fraction was mock-treated, but with addition of guanidine and actinomycin D (*11*). At various times later, an equal number of cells from each experimental protocol was assayed for CAT activity.

In pSV2CAT, the CAT-coding sequence is preceded by a short prokaryotic leader sequence, but is capped by virtue of the eukaryotic transcription initiation process. Cells transfected with pSV2CAT accumulated no further CAT activity after poliovirus infection and even lost some CAT, an indication that CAT translation from this mRNA proceeds in a cap-dependent manner (Fig. 2A and line 1 of Table 1).

By contrast, and as would be expected if the poliovirus 5′ noncoding region can direct cap-independent translation, CAT activity was strongly stimulated by poliovirus infection in cells transfected with a construct in which the wild-type poliovirus 5′ noncoding region preceded the CAT coding sequence (construct pWT-CAT) (Fig. 2B and line 2 of Table 1). The same result was obtained when the leader sequence was from mutant 5NC-11, which contains a mutation that does not affect the translation of the viral RNA but impairs its replication (construct pPN-3) (*7*) (line 3 of Table 1).

With the 5′ noncoding region from three mutants impaired in translation (*7*), the constructs showed a reduced baseline level of CAT activity compared to those with a wild-type sequence. But these mutant 5′ noncoding regions retained some ability to translate in a cap-independent manner because their translation was stimulated by poliovirus infection (Fig. 2C and lines 4, 6, and 7 of Table 1).

Most revealing were mutations that we had previously identified as lethal when

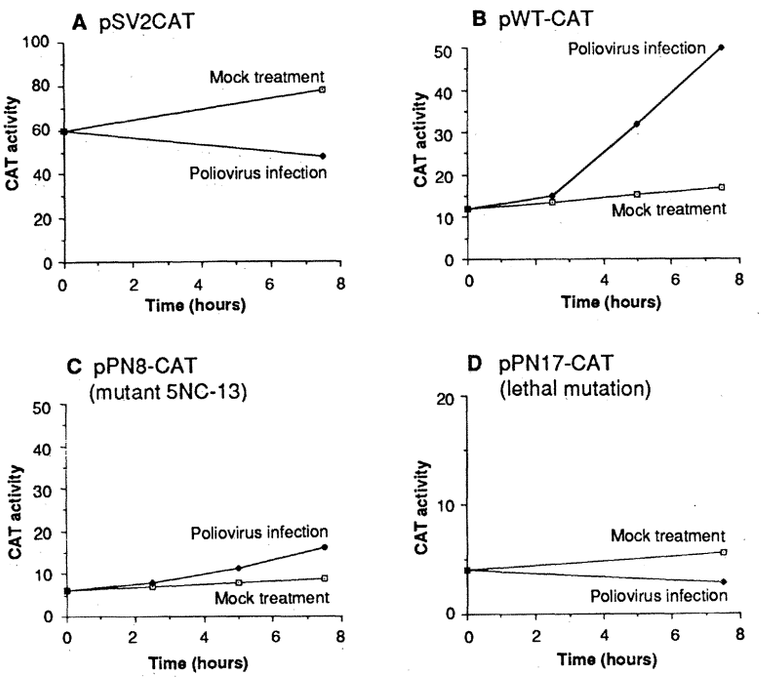

Fig. 2. Effect of poliovirus infection on the expression of a gene placed downstream from different 5′ noncoding regions. COS cells were electroporated with constructs containing the CAT coding sequence downstream from various 5′ noncoding regions and either mock-treated or infected with poliovirus 40 hours later (time 0) (*10*). Cytoplasmic extracts were assayed for CAT activity at various times later, as described (*17*).

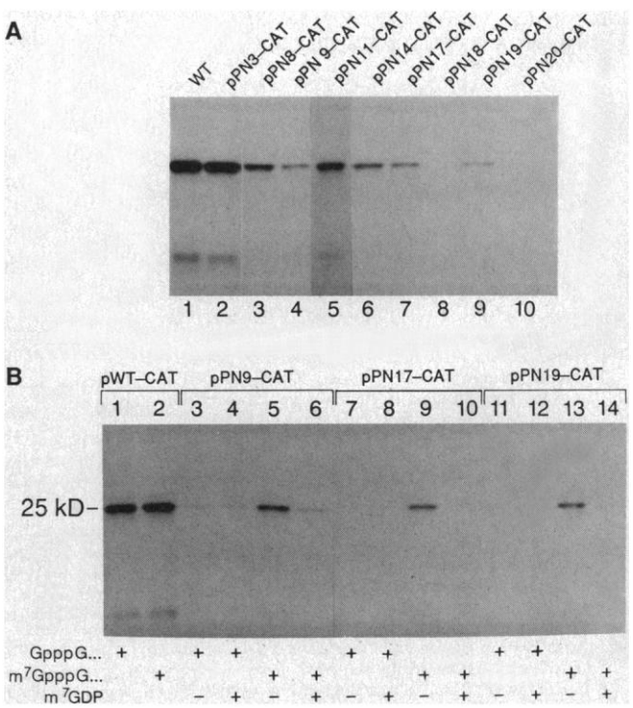

Fig. 3. In vitro translation experiments. (**A**) Translation of wild-type and mutated poliovirus 5′ noncoding region CAT mRNAs in mock-infected HeLa cell extracts. Translations of RNAs synthesized in vitro were performed as described (*8*); mRNA concentrations of 36 μg/ml were used. The major band corresponds to the expected polypeptide of 25 kD; the second polypeptide of 16 kD probably arises from initiation at a downstream in-frame AUG codon that is expected to yield a polypeptide of this size (*9*). The stability of the different mRNAs in HeLa cell extracts was checked and found to be similar for all. (**B**) Effect of cap analog on translation of mRNAs derived from pWT-CAT and constructs with lethal mutations in mock-infected extracts (*8*). Lanes corresponding to unmethylated (GpppG. . .) and methylated (m^7GpppG. . .) capped mRNAs are indicated; m^7GDP (0.1 m*M*) was added where indicated.

introduced into full-length clones (for these mutants, no infectious virus particles could be recovered from transfected cells) (7). Constructs with such mutations lost the potential to translate in a cap-independent manner (Fig. 2D and line 5 and lines 8 to 11 of Table 1). With these constructs, the baseline level of translation was not null, but poliovirus infection had no stimulating effect on translation. We interpret the lack of stimulation as implying no ability to translate in a cap-independent manner. We attribute the baseline activity to the fact that the mRNA from all constructs was capped, being made from an SV40 promoter. Therefore, some cap-dependent translation can take place (see below).

As a confirmation of the in vivo results, we measured the translational ability of the various constructs in vitro. The CAT mRNAs were synthesized with T7 phage RNA polymerase and were translated in extracts from mock- or poliovirus-infected HeLa cells, as previously described (*8*) (Fig. 3) (only results from mock-infected cell extracts are shown). The construct with the wild-type poliovirus 5′ noncoding region stimulated translation efficiently (lane 1 of Fig. 3A), and its activity was independent of whether or not the RNA was capped (lanes 1 and 2 of Fig. 3B). The mutant defective in RNA synthesis was equally active (lane 2 of Fig. 3A). Constructs corresponding to viable region P mutants (7) showed a dramatic reduction in their translational ability compared to wild type (lanes 3, 5, and 6 of Fig. 3A). Constructs with lethal mutations translated even more poorly (lane 4 and lanes 7 to 10 of Fig. 3A). In poliovirus-infected cell extracts, constructs from viable mutants showed a pattern similar to the one observed in mock-infected cell extracts, By contrast, no translation was obtained from constructs with lethal mutations. Furthermore, it was confirmed that the residual translation observed in vivo with this latter class of RNAs occurs in a cap-dependent manner; translation of methylated CAT mRNA made from these constructs was an average of fivefold better than that of its unmethylated counterpart (Fig. 3B; compare lanes 5, 9, and 13 to lanes 3, 7, and 11, respectively); the slight translation of the unmethylated CAT mRNA was not affected by the addition of 7-methylguanosine diphosphate (m^7GDP), a specific inhibitor of the cap-dependent translation of methylated capped RNA in vitro (*12*) (lanes 4, 8, and 12 of Fig. 3B); and addition of m^7GDP to methylated CAT mRNA reduced its translation to the level observed with the unmethylated CAT mRNA (lanes 6, 10, and 14 of Fig. 3B). The poliovirus 5′ noncoding region can therefore support cap-dependent translation if it is part of a capped mRNA. One might have predicted the contrary, as no less than eight AUGs are present in this sequence. The possibility still exists, however, that such a cap-dependent translation can happen only when cap-independent translation is abolished by crucial mutations.

Strikingly, the 5′ noncoding region of all viable region P mutant viruses we had obtained previously had kept the ability to initiate translation in a cap-independent manner, whereas none of the clones tested that originally contained lethal mutations had this ability. This implies that region P is absolutely required for significant translation of poliovirus RNA.

Our combined in vivo and in vitro studies show directly that a long segment of the 5′ end of poliovirus RNA has a role in allowing this mRNA to initiate translation in a cap-independent manner. Region P functions independently of the rest of the viral RNA because it can be appended to a reporter gene and will confer on it cap independence. Region P extends from about nucleotide 130 to about nucleotide 600; its exact extent is being determined. Although we have no direct knowledge of the mechanism by which it functions, the abolition or modification of its function by numerous small alterations suggests that it probably forms a large coherent structure. Nonfunctional mutants include such minimal changes as two- or three-base deletions at positions 460 or 499 (pPN-17 or pPN-19), respectively. The sequence from four revertants of a mutant Sabin 1 poliovirus, made by introducing the same four bases as in mutant 5NC-13, has been determined; these revertants contained the original insertion, but had compensatory point mutations at positions 186 and 525 in three cases and at positions 186 and 480 in the fourth one (*13*). These observations and data obtained from stepwise deletions in region P (*14*) suggest that this functional unit cannot be considered in a linear fashion. Secondary structure must play a role.

Because all picornaviruses translate their genome in a cap-independent manner, it is likely that all of their 5′ noncoding regions would behave similarly in this experimental model. The 5′ end of other viral RNAs such as alfalfa mosaic virus type 4 mRNA, which is able to translate in poliovirus-infected HeLa cells (*15*), would probably show similar results. Cellular mRNAs exist that have unusually long 5′ untranslated regions (*16*). Whether some of them can direct cap-independent translation can be tested with the system described here.

Although the function of region P is still

Table 1. Translation of 5NC-CAT constructs in COS cells. All of the constructs contain a CAT gene cloned at position 630 of wild-type or mutated poliovirus noncoding region. Mutations given by pPN correspond to the mutated clones described in (7). The chart at the bottom of the table gives their location on poliovirus RNA. "Corresponding virus" represents the phenotype of viruses obtained through transfection of HeLa cells with full-length poliovirus cDNA containing the corresponding mutation; R denotes a viable mutant primarily defective in RNA synthesis; P indicates a viable mutant with impairment of viral protein synthesis; and D indicates lethal mutations (no infectious virus particle obtained from transfected cells). Names in parentheses correspond to those of the mutant viruses (7). CAT activity is shown 40 hours after electroporation; the activity obtained with pWT-CAT, which contains a wild-type poliovirus 5′ noncoding region, is taken as reference. Changes in CAT activity are recorded 7½ hours after mock treatment and 7½ hours after poliovirus infection. The experiment was repeated several times and gave highly reproducible results.

Construct	Corresponding virus	CAT activity		
		40 hours after electroporation	7½ hours after mock infection	7½ hours after poliovirus infection
1) pSV2-CAT		5	×1.3	×0.8
2) pWT-CAT		1	×1.4	×3.8
3) pPN3-CAT	R (5NC-11)	0.90	×1.4	×4.1
4) pPN8-CAT	P (5NC-13)	0.50	×1.5	×2.7
5) pPN9-CAT	D	0.26	×1.6	×1.3
6) pPN11-CAT	P (5NC-114)	0.75	×1.2	×2.1
7) pPN14-CAT	P (5NC-116)	0.70	×1.4	×3.1
8) pPN17-CAT	D	0.30	×1.4	×0.7
9) pPN18-CAT	D	0.21	×1.2	×1.0
10) pPN19-CAT	D	0.29	×1.6	×0.9
11) pPN20-CAT	D	0.40	×1.4	×0.9

Nucleotide number	70	224	270	392	460	499
	pPN3	pPN8 pPN9	pPN11	pPN14	pPN17 pPN18	pPN19 pPN20

to be defined, it is easiest to imagine a cis-acting role. It might, for instance, provide an entry site for ribosomes onto the mRNA, either by being a binding site or by assisting ribosomes to enter downstream from itself. However, a trans-acting process, such as ribosome modification, cannot be completely ruled out. Region P could even perform functions the ribosome itself normally accomplishes to initiate translation.

It is evident that region P of poliovirus can direct mammalian cells to translate mRNA in a cap-independent fashion. Poliovirus infection or poliovirus protein 2A expression can stop cellular cap-dependent protein synthesis, freeing ribosomes that will then translate only region P–containing RNAs. Thus, it should be possible to use region P and protein 2A to focus cellular translation on a few chosen mRNAs and increase the yield of specific proteins made in mammalian cells.

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Carboxyl Terminal Domain of $G_{s\alpha}$ Specifies Coupling of Receptors to Stimulation of Adenylyl Cyclase

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The α subunits of G_s and G_i link different sets of hormone receptors to stimulation and inhibition, respectively, of adenylyl cyclase. A chimeric α_i/α_s cDNA was constructed that encodes a polypeptide composed of the amino terminal 60% of an α_i chain and the carboxyl terminal 40% of α_s. The cDNA was introduced via a retroviral vector into S49 *cyc*$^-$ cells, which lack endogenous α_s. Although less than half of the hybrid α chain is derived from α_s, its ability to mediate β-adrenoceptor stimulation of adenylyl cyclase matched that of the normal α_s polypeptide expressed from the same retroviral vector in *cyc*$^-$ cells. This result indicates that carboxyl terminal amino acid sequences of α_s contain the structural features that are required for specificity of interactions with the effector enzyme, adenylyl cyclase, as well as with the hormone receptor.

MANY MAMMALIAN SIGNAL TRANSduction pathways use a G protein to couple hormone receptors to effector molecules. Upon interaction with an activated receptor, the α polypeptide chains of G proteins undergo a guanosine triphosphate (GTP)–dependent conformational change that allows them to activate effector enzymes or open ion channels (*1, 2*). Each member of the α chain family interacts specifically with receptors and effectors. Extensive conservation of primary structure among different G protein α chains (*1, 2*) suggests that topologically equivalent portions of each α chain may subserve the same functions in each, namely, binding and hydrolysis of guanine nucleotide, and interactions with receptors and effectors. In a recently constructed model (*3*) of a composite G protein α chain (α_{avg}), we tentatively assigned these functions to different regions of α_{avg}. In our model (*3*), regions of linear sequence that presumably contribute to the polypeptide's guanine binding site divide the rest of the chain into three potential domains. Biochemical evidence (*1, 3*) strongly suggests that the extreme COOH-terminus (domain III) directly contacts receptors. This conclusion is supported by the identification (*4*) of a mutation, located in the same region of α_s, that specifically uncouples G_s from receptors. We proposed (*3*) that domain II interacts with effectors, on the basis of studies (*5, 6*) that suggested similar roles for the corresponding regions of two other GTP-binding proteins, bacterial elongation factor Tu (EF-Tu) and the mammalian 21-kD polypeptides (p21) encoded by the *ras* oncogenes.

The high degree of conservation among nucleotide sequences of different α chains provided a simple approach to testing these functional assignments. A conserved Bam HI restriction endonuclease site, present in almost all mammalian α chain cDNAs, neatly separates domains I and II from domain III. This site allows convenient construction of chimeric cDNAs in which a hybrid polypeptide derived from two different α chains is encoded in a single reading frame. The first chimeric α chain we constructed (Fig. 1) encodes the NH_2-terminal 212 amino acids of a murine α_i chain (7), designated α_{i2} (*8*), linked to the COOH-terminal 160 residues of murine α_s (7). Whether α_{i2} is the α chain of a G protein that mediates hormonal inhibition of adenylyl cyclase is unknown. If so, the postulated locations of receptor- and effector-recognition sites predict that the chimera would couple β-adrenoceptors (interacting with domain III of the α_s portion of the chimera) to inhibition of adenylyl cyclase (mediated by the postulated effector interaction region in domain II, contributed by α_{i2}).

To investigate the function of the α_i/α_s chimera, we expressed it in S49 *cyc*$^-$ by means of a retroviral vector as previously described (*4*). We compared regulation of

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